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Increased Ubiquitin Immunoreactivity in Unstable Atherosclerotic Plaques Associated With Acute Coronary Syndromes

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OBJECTIVES The current study was designed to examine whether ubiquitin expression is higher in unstable than in stable lesions of patients with acute coronary syndrome (ACS).

BACKGROUND The ubiquitin system has been identified as the nonlysosomal pathway of protein degradation; it is involved in a number of biologic processes crucial to cell and tissue integrity and therefore, might be potentially involved in the rupture of unstable coronary plaques.

METHODS We conducted an autopsy-based study of 25 consecutive patients with fatal ACS. Lesions of both infarct-related and noninfarct-related segments from the same patients were examined for the expression and localization of ubiquitin by use of immunohistochemistry and a semiquantitative grading scale.

RESULTS Ubiquitin immunoreactivity was higher in infarct-related than in noninfarct-related lesions (1.4 ± 0.5 vs. 1.1 ± 0.6, $p = 0.03$). Compared with areas adjacent to the plaque (0.6 ± 0.7), ubiquitin immunoreactivity was higher in areas around the lipid core (2.5 ± 0.8, $p < 0.001$), plaque shoulders (1.6 ± 1.1, $p < 0.001$), and fibrous cap regions (1.6 ± 1.1, $p < 0.001$). Within the plaque area, co-localization of ubiquitin immunoreactivity with T cells and macrophages was found. In areas adjacent to the plaque, ubiquitin immunoreactivity co-localized with neointima cells and media smooth muscle cells.

CONCLUSIONS In patients with ACS, ubiquitin immunoreactivity is enhanced in unstable, infarct-related lesions, predominantly in plaque regions of tissue degradation. Based on these findings, this study suggests a role for the ubiquitin system in the destabilization and rupture of coronary atherosclerotic plaques in humans. (J Am Coll Cardiol 2002;40:1919–27) © 2002 by the American College of Cardiology Foundation

Atherosclerosis research has revealed molecular mediators and mechanisms involved in the atherosclerotic process (1,2). Notably, inflammation and matrix protein degradation have been associated with the rupture of unstable coronary plaques, resulting in acute coronary syndrome (ACS) (3–6).

The ubiquitin-mediated proteolytic pathway involves the conjugation of multiple moieties of ubiquitin, a 76-amino-acid polypeptide, to cellular proteins in a multienzymatic process, targeting these proteins to degradation (7,8). As the majority of cellular proteins are affected by this nonlysosomal pathway of protein degradation, the ubiquitin system has been identified as crucially involved in a variety of biologic processes, maintaining cell and tissue integrity (7). Thus, it may conceivably play a role in the process of coronary plaque instability and rupture. The current study has been designed to test whether ubiquitin expression is higher in unstable than in stable lesions of patients with ACS.

METHODS

Study population. Approved by the Institutional Review Board, the study included 25 consecutive patients with fatal ACS, in which, acute or subacute myocardial infarction (MI) was confirmed by autopsy at the Mayo Clinic Rochester during 1995 and 1996 (9–11). In addition, five patients with noncardiac death were included as a reference group.

After gross evaluation, sections were taken from anterior, lateral, and inferior regions of the left and right ventricles at basal, middle, and inferior regions. Presence and age of MI were determined according to previously published criteria (12). Myocardial infarctions were designated as transmural if they extended >50% of wall thickness, as subendocardial if they extended <50% of wall thickness, and as mixed if a combination of both was present.

At autopsy, coronary arteries were fixed in 10% neutral buffered formalin. Subsequently they were embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin-eosin and Verhoeff-van Gieson (13).

Immunohistochemistry. As described in detail before (14), after deparaffinization and rehydration, endogenous peroxidase activity and nonspecific protein binding sites were blocked by equimolar H_2O_2 methanol solution and 5% goat, respectively. Anti-ubiquitin primary antibody (Sigma, St. Louis, Missouri) was applied at a dilution of 1:100 at 4°C overnight. Biotinylated secondary antibody (rabbit IgG, dilution 1:400, Dako, Carpinteria, California) was detected by peroxidase-conjugated streptavidin (dilution 1:500, Dako) and visualized by 3-amino-9-ethylcarbazole (Sigma).

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Abbreviations and Acronyms

ACS = acute coronary syndrome
LDL = low density lipoprotein
MI = myocardial infarction
RT = room temperature
TdT = terminal deoxynucleotidyl transferase
TUNEL = terminal deoxynucleotidyl transferase end labeling

Tissue cell types were identified by use of anti-smooth muscle alpha-actin (dilution 1:1500, Dako), anti-macrophage KP 1 (dilution 1:200, Dako), and anti-T cell CD 3 (dilution 1:200, Dako). Expression of p53 was assessed by a monoclonal antibody (dilution 1:100, Cell Signaling Technology, Inc., Beverly, Massachusetts). All sections were counterstained with hematoxylin.

Terminal deoxynucleotidyl transferase end labeling (TUNEL) assay. Cells undergoing apoptosis were identified by the (TdT) end labeling TUNEL technique using the ApopTag In Situ Apoptosis Detection Kit from Intergen (Intergen Company, Purchase, New York). Briefly, deparaffinized and hydrated sections were digested with 20 μg/ml proteinase K for 11 min at room temperature (RT), followed by rinsing and permeabilization of tissue by incubation with 0.05% triton $\times-100$ in 0.05% sodium citrate for 5 s. After rinsing, endogenous peroxidase activity was quenched by incubation with 1.5% H_2O_2 at RT for 15 min. Sections were successively incubated with equilibration buffer (5 min at RT), terminal deoxynucleotidyl transferase (TdT) enzyme (60 min at 37°C), stop/wash buffer (10 min at RT), and protein block (Dako) (7 min at RT). After incubation with anti-digoxygenin peroxidase at RT for 30 min and rinsing, slides were incubated with NovaRed substrate for 2 min at RT and counterstained with hematoxylin. Female rodent mammary gland tissue, three to five days after weaning, was used as a positive control, assuring a rate of apoptotic cells of 1% to 2% (provided by Intergen Company, Purchase, New York) (15). Omission of TdT enzyme from the labeling procedure served as a negative control (16).

Double-label immunohistochemistry. To further identify cell types expressing ubiquitin immunoreactivity, double-label immunostaining of specimens was performed using the EnVision Doublestain Kit (Dako) with the specific antibodies mentioned previously. In the first step, the ubiquitin antibody was detected by a peroxidase-labeled secondary antibody with 3,3-diaminobenzidine tetrahydrochloride as chromogen (Vector Laboratories, Inc., Burlingame, California) to yield a brownish reaction product. In the second step, cell-specific antibodies were detected by an alkaline phosphatase-labeled secondary antibody with Vector Red as chromogen (Vector Laboratories). Endogenous alkaline phosphatase was blocked by incubation with levamisole (Dako).

Table 1. Study population

Number of Patients	25
Cardiovascular risk factors	
Male gender	13 (52%)
Hyperlipidemia	7 (28%)
Systemic hypertension	14 (56%)
Smoking	7 (28%)
Obesity	9 (36%)
Family history	1 (4%)
Diabetes mellitus	8 (32%)
Cardiac history	
Prior percutaneous coronary intervention	8 (32%)
Prior coronary artery bypass grafting	1 (4%)
Prior acute MI	15 (60%)
Congestive heart failure	10 (40%)
Clinical diagnosis	
Acute MI	21 (84%)
Unstable angina pectoris	0 (0%)
Sudden cardiac death	4 (16%)
Pathologic diagnosis	
MI <24 h	8 (32%)
MI >24 h	17 (68%)
Subendocardial MI	8 (32%)
Transmural MI	9 (36%)
Mixed MI	7 (28%)

Values are n (%).
MI = myocardial infarction.

Histologic analysis. Coronary artery specimens were analyzed by light microscopy, and atherosclerotic lesions were defined according to the criteria set forth by the Committee on Vascular Lesions of the Council on Arteriosclerosis of the American Heart Association (3). Plaque areas were stratified into (Ia) intima and (Ib) media adjacent to the plaque, (II) shoulder region, (III) fibrous cap, and (IV) lipid core as described by Jonasson et al. (17). In these areas grading of ubiquitin immunoreactivity was performed by a semiquantitative scale, defining absence of staining as Grade 0, weak intensity as Grade 1, intermediate intensity as Grade 2, and strong intensity as Grade 3 (Fig. 1). From the values obtained for these regions, mean ubiquitin staining grades were calculated for the plaque area itself (regions II, III, and IV), for the area adjacent to the plaque (regions Ia and Ib), and for all regions of each lesion. Following completion of analyses, performed by one observer, data were stratified according to plaque grade for the entire population and according to infarct-related and noninfarct-related artery for one patient. The average coefficient of correlation between five repetitive gradings of specimen ubiquitin immunoreactivity was 0.812 ± 0.032. The average mean difference between five repetitive gradings of specimen ubiquitin immunoreactivity was 0.121 ± 0.100.

Statistical analysis. Continuous data were expressed as mean $\pm$ SD, and categorical data were expressed as percentages. All comparisons of ubiquitin staining grades between the regions of infarct-related and noninfarct-related lesions of the same patient, as well as between the different regions on lesion level, were made by signed-rank

Figure 1. Illustration of the immunostaining grades applied in the current study. Original magnifications for all panels 37.5×.

test. A p value <0.05 was considered statistically significant for all analyses.

RESULTS

Study population. Demographic data for the study population are presented in Table 1. Myocardial infarction to death interval was 4.8 ± 3.9 days, and death to autopsy interval was 12.4 ± 6.3 h. All patients had a history of multivessel coronary artery disease.

Histology. The left anterior descending artery, the left circumflex artery, and the right coronary artery was the infarct-related artery in 13 (52%), 4 (16%), and 8 (32%) of the cases, respectively. Among the infarct-related coronary arteries, the Stary grade of the underlying lesion morphology was Stary IV, Stary V, Stary VII, and Stary VIII in 2 (8%), 11 (44%), 9 (36%), and 3 (12%) of the cases, respectively. A surface defect was observed in 18 cases (68%) and isolated thrombus in 7 cases (32%). Among the noninfarct-related coronary arteries, Stary grade V, VII, and VIII were found in 7 (28%), 14 (56%), and 4 (16%) of the cases, respectively.

Immunoreactivity. Ubiquitin immunoreactivity was significantly higher in infarct-related than in noninfarct-related lesions (1.4 ± 0.5 vs. 1.1 ± 0.6, $p = 0.03$), which was attributable mainly to greater staining within the plaque area (2.3 ± 0.6 vs. 1.5 ± 0.7, $p < 0.001$) rather than outside the plaque area (0.5 ± 0.7 vs. 0.8 ± 0.7, $p = 0.09$). Specifically, ubiquitin immunoreactivity was higher in the infarct-related lesions compared with the noninfarct-related lesions in the shoulder region (2.1 ± 0.9 vs. 1.0 ± 0.9, $p < 0.001$) and in the region of the fibrous cap (2.2 ± 0.9 vs. 1.0 ± 1.0, $p < 0.001$), but not in the area around the lipid core (2.6 ± 0.7 vs. 2.3 ± 0.9, $p = 0.3$). No significant difference in ubiquitin immunoreactivity between infarct-related and noninfarct-related lesions was found in the intima (0.6 ± 0.8 vs. 1.0 ± 0.9, $p = 0.1$) or in the media (0.3 ± 0.6 vs. 0.6 ± 0.7, $p = 0.2$). Figure 2 gives a representative immunohistologic example.

Overall, ubiquitin immunoreactivity was higher around the lipid core (2.5 ± 0.8) compared with the shoulder (1.5 ± 1.1, $p < 0.001$) and cap region (1.6 ± 1.1, $p < 0.001$). In areas adjacent to the plaque, immunoreactivity was higher in the intima than in the media (0.8 ± 0.9 vs. 0.4 ± 0.7, $p < 0.001$) because of intense staining of cells at the base of the neointima.

In less diseased (Stary II and Stary III) coronary artery specimens from patients with noncardiac death, specific ubiquitin staining was absent.

Tissue ubiquitin immunoreactivity was absent when the slides were incubated with an unspecific isotype antibody (rabbit IgG) or when recombinant ubiquitin was added to the anti-ubiquitin antibody (ratio 2:1).

Co-localization. Immunostaining in serial sections indicated co-localization of ubiquitin immunoreactivity with neointimal cells and inflammatory cells in cap and shoulder regions (Fig. 2C to 2F). Double immunostaining confirmed co-localization of ubiquitin immunoreactivity with T cells in areas of inflammatory cell infiltration (Fig. 3) and with macrophages in lipid core regions (Fig. 4). Co-localization of ubiquitin immunoreactivity with TUNEL-positive cells was seen in lipid core as well as cap/shoulder regions (Fig. 5). In these regions, ubiquitin immunoreactivity co-localized with p53 as well (Fig. 6). In the region adjacent to the plaque, ubiquitin immunoreactivity co-localized with neointimal cells and media smooth muscle cells.

DISCUSSION

The current study demonstrates high ubiquitin immunoreactivity in unstable coronary plaques in patients with ACS, consistent with a potential role for the ubiquitin system in

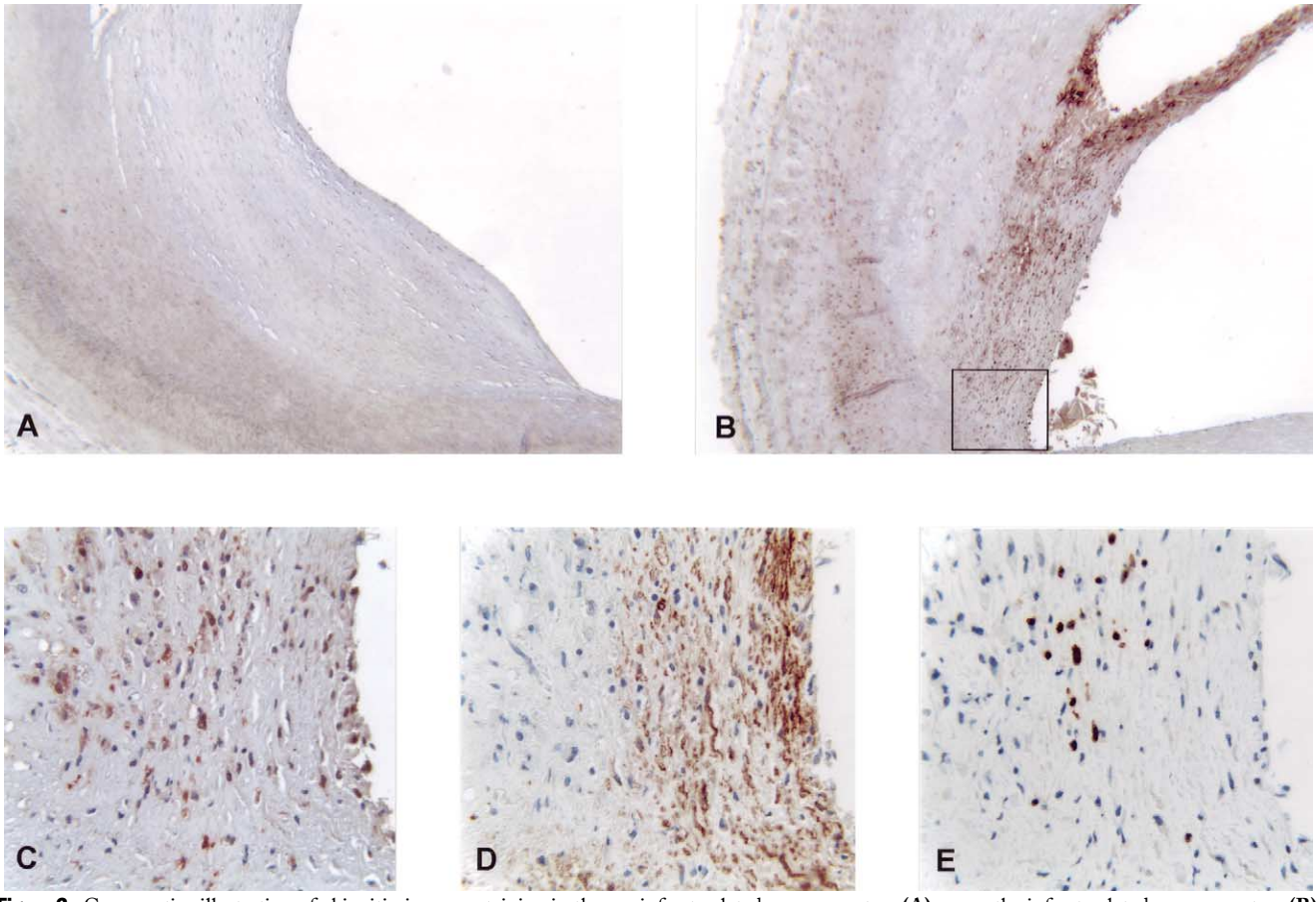

Figure 2. Comparative illustration of ubiquitin immunostaining in the noninfarct-related coronary artery **(A)** versus the infarct-related coronary artery **(B)**. The **area highlighted by the box in B** is displayed at a higher magnification in **C.** Serial sections stained for smooth muscle alpha-actin **(D)** and T-cell CD 3 **(E)** reveal co-localization of ubiquitin immunoreactivity with inflammatory cells and neointimal cells in the cap/shoulder region. Original magnifications for **A and B** 15×; for **C to D** 100×.

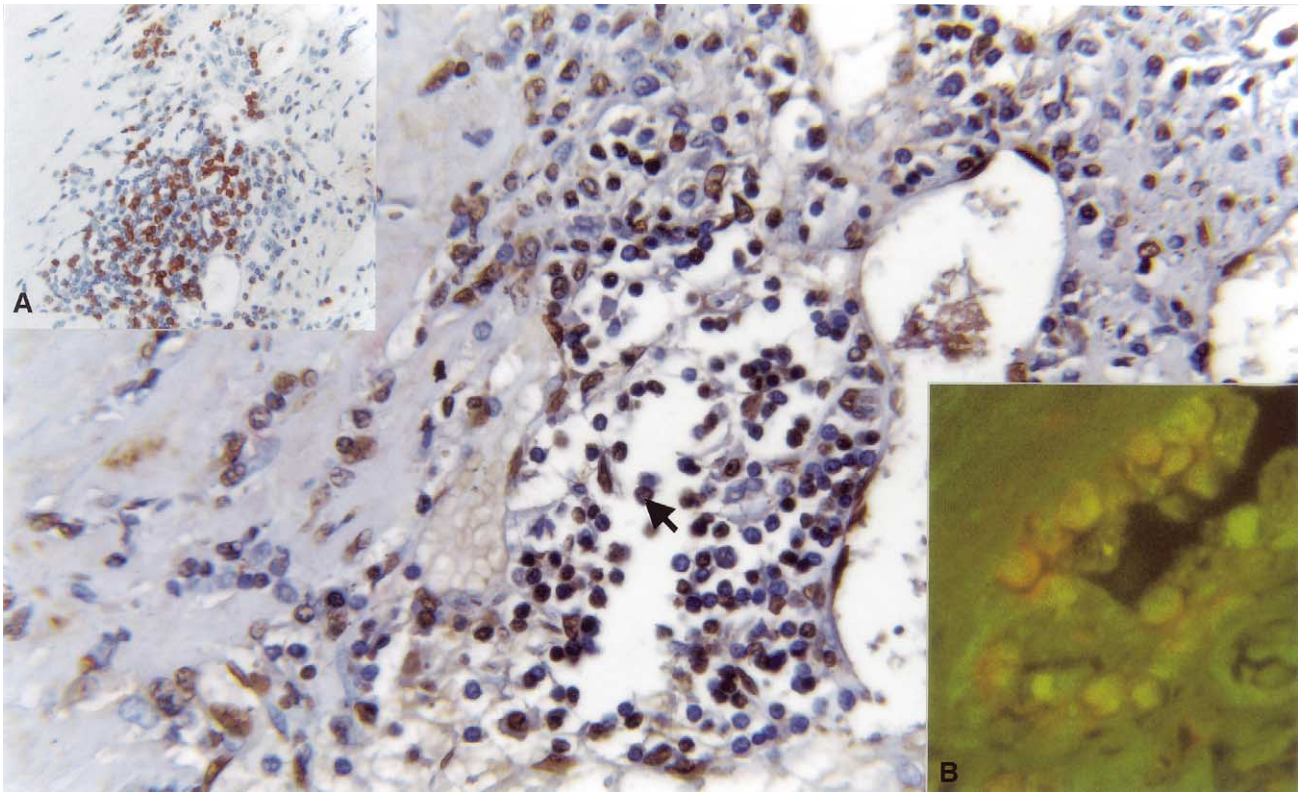

Figure 3. Atherosclerotic plaque area characterized by infiltration of leukocytes, most of them revealing **brown** staining for ubiquitin **(arrow in main panel)** and being identified as T cells by CD-3 immunostaining **(A)**. Double immunofluorescence confirms co-localization of ubiquitin with T cells (round cells with **yellow** fluorescence for ubiquitin in the center, surrounded by **red** fluorescence for CD-3, **B**). Original magnification for all panels 100×.

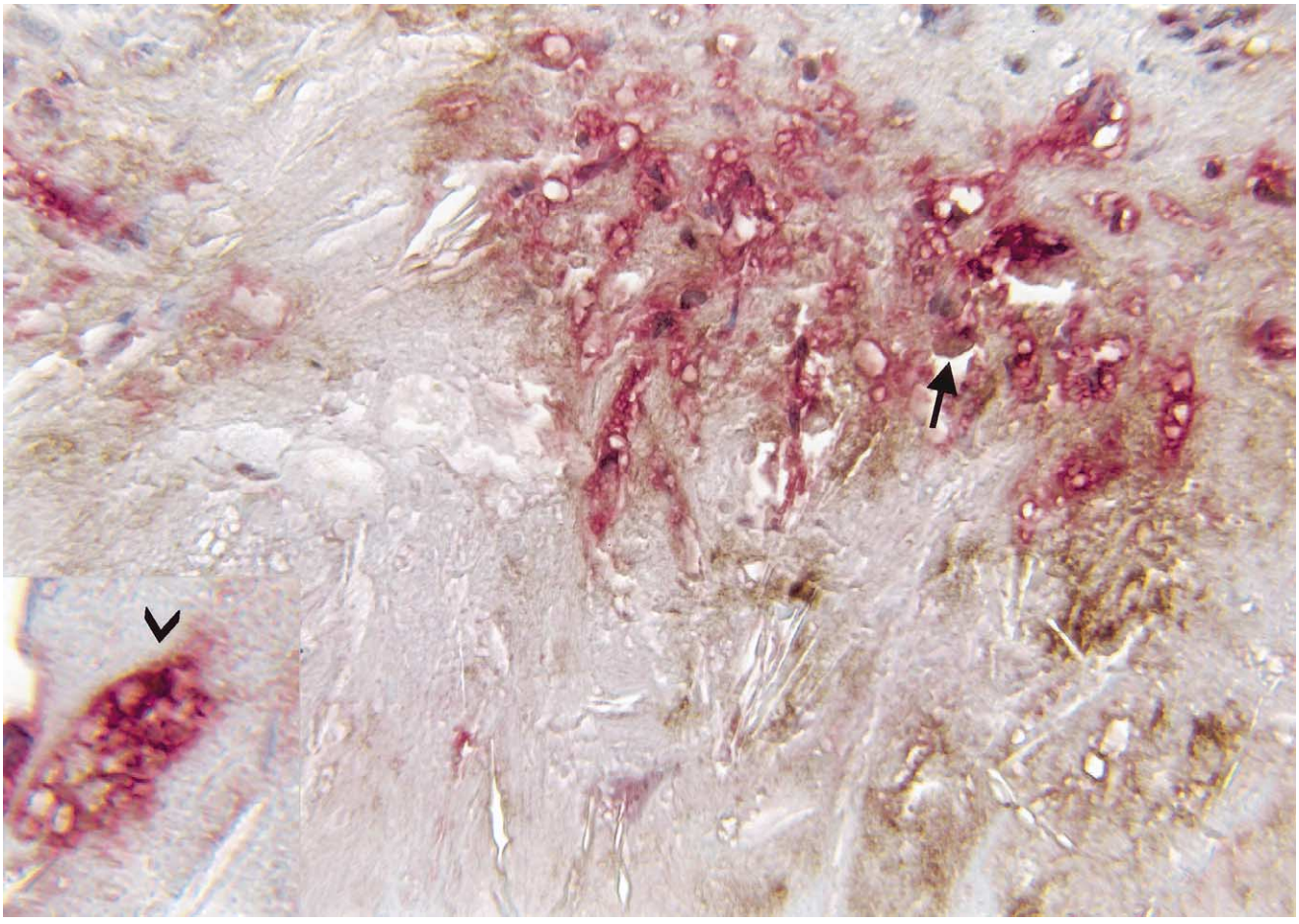

Figure 4. Lipid core area of an atherosclerotic plaque, in which co-localization of ubiquitin **(brown)** with macrophage CD 68 **(red)** is revealed by double-immunostaining **(arrow in main panel)**, including nuclear staining of a foam cell **(arrowhead in inserted panel)**. Original magnification for both panels 100×.

progression and acute complication of coronary atherosclerosis in humans.

Previous pathologic studies have identified a small fibrous cap, formed by extracellular matrix components, and a large necrotic core, formed by cellular and lipid debris, as the characteristic morphology of unstable atherosclerotic plaque (3,4). Indeed, most of the infarct-related lesions in the current study exhibited this morphologic pattern and were also characterized by infiltration of inflammatory cells (5). Among them, T cells are regarded as a cell population of quantitative and qualitative significance for the activity of the inflammatory process. Notably, previous in-vitro studies highlighted the involvement of the ubiquitin-proteasome system in the metabolism of T cell antigen receptor subunits, affecting their assembly and function (18,19). These experimental findings might explain the intense ubiquitin immunoreactivity displayed by T cells in the current immunohistologic study. Thus, ubiquitination can be localized to T cells in regions of inflammatory cell infiltration of atherosclerotic plaques, with the potential not only to affect T cell function but overall inflammatory activity and plaque stability.

Another area of strong ubiquitin immunoreactivity identified in the current study is the lipid core region, rich in modified low density lipoprotein (LDL) and their degradation products (20). The important role for the ubiquitin system in the degradation of oxidized proteins has been known for years (21). Of note, it has been shown that oxidative stress can, in fact, stimulate the ubiquitin system in macrophages by inducing the expression of components of its enzymatic machinery such as ubiquitin-binding proteins (22). Furthermore, it has been demonstrated that aggregated LDL induces the expression of the human homologue of the bovine ubquitin-conjugating enzyme E2-25K in human monocytes, which was associated with polyubiquination of intracellular proteins (23). As p53 was identified as one of the proteins undergoing ubiquitin-dependent degradation, it was concluded that aggregated LDL might contribute to foam cell formation by stimulation of the ubiquitin-proteasome pathway and subsequent degradation of pro-apoptotic proteins. Oxidized LDL might have the same effect, although in high concentration it can, in fact, inhibit the proteolytic activity of the proteasome (24). Given unimpaired ubiquitination, this would lead to the accumulation of ubiquitinated proteins and increase in the half-life of substrates of the ubiquitin-

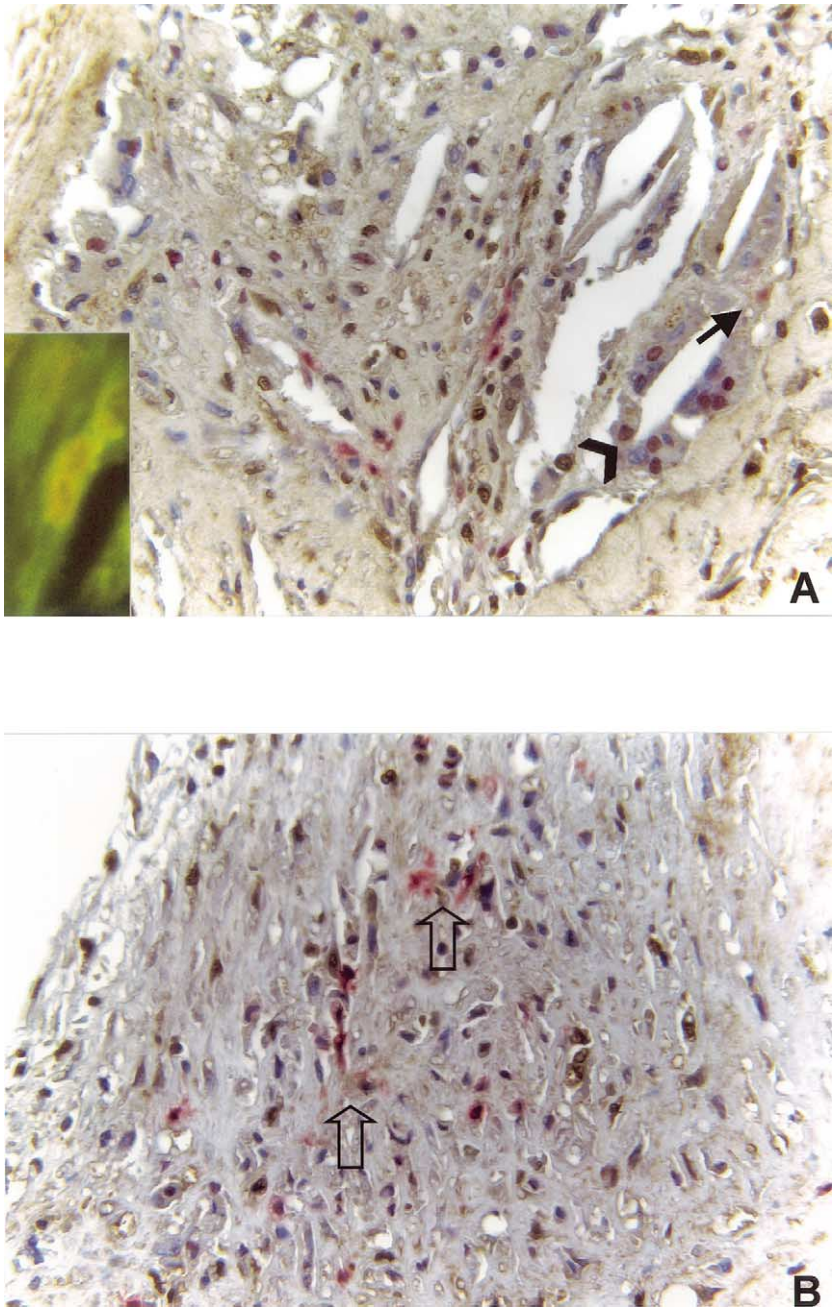

Figure 5. Co-localization of ubiquitin **(brown)** with terminal deoxynucleotidyl transferase end labeling (TUNEL) positive cells **(red)** in lipid core area **(A)** and cap/shoulder region **(B)**. Double immunofluorescence **(inserted panel)** more clearly visualizes ubiquitin (**yellow** fluorescence) in the cytoplasm of cells, which line cholesterol crystals of the lipid core and stain positive for TUNEL (**red** fluorescence). Original magnification for all panels 100×.

proteasome system such as p53 (24,25). Indeed in a previous study on carotid atherectomy samples accumulation of p53 was demonstrated in all cell types of the plaque, but mainly confined to macrophages and vascular smooth muscle cells (26). Importantly, p53 accumulation localized to the same compartments of the plaque as positive TUNEL staining, mainly around the atheromatous gruel (26). In line with these results, p53 immunoreactivity was found mainly in the area of the lipid core and, to a lesser extent, in the cap/shoulder region. In the present study, p53 immunoreactivity was found mainly with macrophages and, to a lesser extent, with smooth muscle cells and T cells. Of further note, p53 staining co-localized with ubiquitin immunoreactivity in the very same plaque area in which TUNEL-positive cells were identified. Therefore, proteasome inhibition with subsequent accumulation of substrates of the ubiquitin-proteasome system has to be considered as another explanation for extensive ubiquitin immunoreactivity in human plaques in vivo. As exemplified for p53, these accumulating substrates of the ubiquitin-proteasome system can trigger the cellular death program, which eventually leads to deoxyribonucleic acid fragmentation as visualized

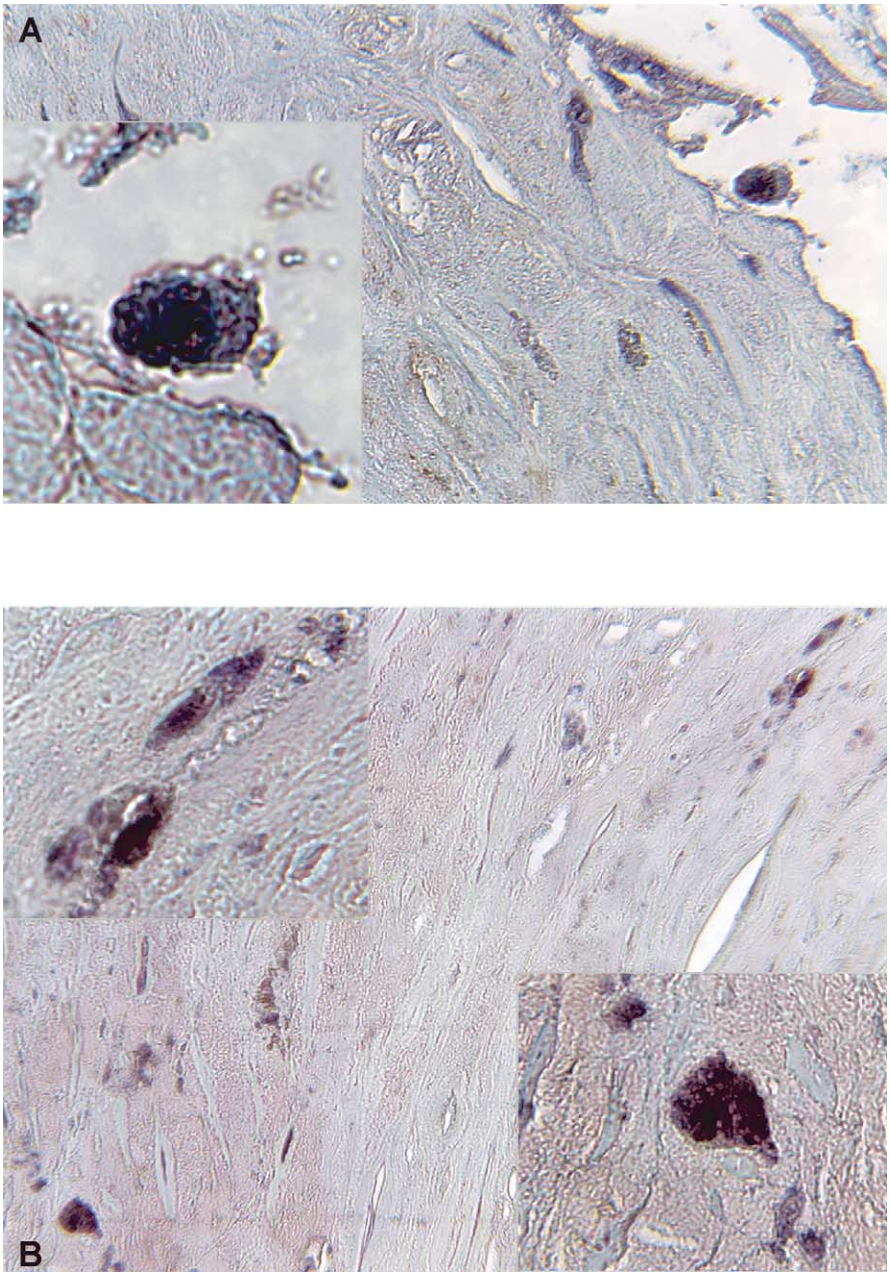

Figure 6. Lipid core region **(A)** and cap region **(B)** revealing co-localization of ubiquitin **(brown)** with p53 **(red)** in macrophages **(inserted panel in A, and lower inserted panel in B)** and vascular smooth muscle cells **(upper inserted panel in B)**. Original magnification for main panels and inserted panels 75× and 150×, respectively.

by positive TUNEL staining. The presence and significance of apoptosis for progression and complication of atherosclerotic plaques has been pointed out before (27–29). Thus, given previous and current study findings, the ubiquitin system can, potentially, be involved in cell survival as well as in cell death in atherosclerosis, with significance for the course of the overall disease process.

In areas adjacent to the plaque, ubiquitin immunoreactivity was confined to media smooth muscle and neointimal cells, whereas immunoreactivity was more pronounced in the neointimal cells. Previously, in situ hybridization of a polyubiquitin riboprobe to sections of diseased human coronary arteries demonstrated higher expression of polyubiquitin in media smooth muscle cells than in neointimal cells (30). An immunohistochemical study on the expression of ubiquitin in normal and injured rat aorta, however, demonstrated intense cellular ubiquitin staining in neointimal regions, and a role for the ubiquitin system in proliferation cell turnover was suggested on the basis of these findings (31). Additionally, enhanced ubiquitin immunoreactivity in these regions might be due to local hypoxia secondary to impairment of oxygen diffusion capacity and even increase in oxygen demand (32,33). Thus, the current study indicates a role for the ubiquitin system in the

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metabolic activity not only of media smooth muscle cells, but also of neointimal cells in the atherosclerotic arterial wall.

Overall, ubiquitin immunoreactivity in advanced atherosclerotic lesions was higher in infarct-related coronary arteries than in noninfarct-related coronary arteries of the same patient in this study, which was attributable to a higher immunoreactivity in the cap and shoulder regions in infarct-related arteries. Previous reports have outlined a pathomechanistic role for inflammation as well as apoptosis in the process of plaque destabilization (1,2,5,28,29). Of note, both these processes have been associated with alterations in the ubiquitin-proteasome system, including increased ubiquitin expression on tissue level (25,34–36). In line with these previous reports, ubiquitin immunoreactivity co-localized with both inflammatory cells and apoptotic cells in cap and shoulder regions in the current study, indicating an involvement of the ubiquitin proteasome system in inflammation and apoptosis with potential impact upon plaque stability. Of note, the cellular target proteins of the ubiquitin-proteasome system in these different biologic processes and cells can differ to various extent. Thus, in nonuniform manner the ubiquitin-proteasome system might be involved in the progression and complication of the atherosclerotic plaque.

Study limitations. One limitation of the current study is its retrospective and descriptive character. However, the pathohistologic approach allowed localization and semiquantitative evaluation of the expression of ubiquitin in a well-defined series of lesions of both infarct-related and noninfarct-related coronary arteries from the same patients in whom the diagnosis of AMI was confirmed by autopsy. Although this study cannot give further mechanistic insight and cannot prove a causative role for the ubiquitin system in plaque instability, it demonstrates the topographics expression of ubiquitin within advanced human atherosclerotic plaques and shows overall higher immunoreactivity of lesions of infarct-related arteries. It has to be noted that plaque composition differed between the two groups, with a higher prevalence of fibrotic and calcified lesions in the group of noninfarct-related coronary arteries; however, this is unavoidable due to the design of the current study. Further studies are warranted to define the functional significance of the enhanced ubiquitin expression in advanced coronary artery lesion associated with acute coronary syndromes. Furthermore, prospective clinical studies would allow sampling of fresh tissue and the application of quantitative methods of the determination of tissue protein expression.

Conclusions. The current study demonstrates enhanced ubiquitin immunoreactivity in unstable, infarct-related plaques, predominantly in plaque regions of tissue degradation. In areas adjacent to the plaque, ubiquitin immunoreactivity was enhanced at the base of the neointima. Based on these findings, the current study suggests a role for the ubiquitin system in progression and complication of coronary atherosclerotic plaques in humans.

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